Title

Feasibility of using bioaugmentation with bacterial strain PM1 for bioremediation of MTBE-contaminated vadose and groundwater environments

Authors

Scow, Kate M
Hristova, Krassimira

Publication Date

2001-09-30

Water Resources Center Final Report (9/30/01)

Title: Feasibility of using bioaugmentation with bacterial strain PM1 for bioremediation of MTBE-contaminated vadose and groundwater environments. W924

Investigators: Kate M. Scow, Professor
Dept. of Land, Air and Water Resources, UC Davis
Ph:530-752-4632; Fx:530-752-1552; email:
kmscow@ucdavis.edu
Krassimira Hristova, Postdoctoral Researcher
Dept. of Land, Air and Water Resources, UC Davis
Ph:530-752-0146; Fx:530-752-1552; email:
krhristova@ucdavis.edu

Key words: MTBE, bioremediation, biodegradation, microorganisms, groundwater

Abstract

Widespread contamination of groundwater by MTBE has triggered the exploration of different technologies for in situ removal of the pollutant. After laboratory studies revealed that bacterial strain PM1 is capable of rapid and complete MTBE biodegradation, the organism was tested in an *in-situ* bioaugmentation field study at Port Hueneme Naval Base, Oxnard, CA. Two small pilot test plots (A and B) located down gradient from an MTBE source were injected with pure oxygen at two depths. One plot (B) was also inoculated with Strain PM1. MTBE concentrations upstream from plots A and B initially varied temporally from 1.5 to 6 mg per L. By six months after treatment began, MTBE concentrations wells downstream from the injection bed substantially decreased in the shallow zone of the groundwater in both plots, even in the absence of Strain PM1. In the deeper zone, downstream MTBE concentrations also decreased substantially in Plot A and to lesser extent in Plot B. Difficulties in delivery of oxygen to the deep zone of Plot B, evidenced by low dissolved oxygen concentrations, are likely responsible for low rates of MTBE removal at that location. We measured the survival and movement of PM1 in groundwater using three different methods for detection of PM1 rDNA sequences: TaqMan quantitative PCR, Denaturing Gradient Gel Electrophoresis (DGGE), and Intergenic Spacer Region (ITS) analysis. A naturally occurring organism with >99% rDNA sequence similarity to Strain PM1 was detected in groundwater collected outside the test plots. Changes in the groundwater microbial community were also monitored over time using ITS, a PCR-based DNA fingerprinting method. The largest differences in the microbial community profiles were observed between the shallow and deep groundwater samples, regardless of whether they were from plot A or B.

Introduction

The fuel additive, methyl tertiary-butyl ether (MTBE), has become a widespread environmental contaminant in the past decade. Since MTBE was introduced to gasoline as an additive in 1988, its production has increased to 17 billion pounds per year and currently comprises up to 15% of some reformulated gasoline (Kirshner, 1995). A recent study by the United States Geological Survey identified MTBE as the second most common volatile organic compound detected in wells monitored in urban areas nationwide between 1985 and 1995 (Squillace et al. 1995 & 1996). In California, for example, approximately 13 thousand sites were shown to have hydrocarbon-impacted groundwater and greater than 10 thousand of these sites were estimated to be impacted by MTBE (Happel et al. 1998). Once MTBE enters the environment, it is considered to be relatively recalcitrant to degradation. MTBE is very water-soluble (48 g/L), thus it is highly mobile in both ground and surface waters and its transport resembles that of conservative tracers used to predict groundwater flow. The pollutant is also moderately volatile (unitless Henry's law constant 0.04) which can lead to redistribution and further contamination of the vadose zone, surface soils and sediments. The Environmental Protection Agency (EPA) lists MTBE as a possible carcinogen, but toxicity limits are a subject of current debate. The compound can be detected by both taste and odor at concentrations as low as 35 ug/L, thus the EPA has recommended keeping MTBE concentrations in drinking water below a 20-40 ug/L nuisance limit.

There is little evidence that extensive intrinsic remediation is occurring at MTBE contaminated sites. Thus it is important to explore the potential of using active bioremediation, a potentially promising technology for inexpensive treatment of MTBE contaminated groundwater. Our laboratory has recently isolated a bacterial culture, Strain PM 1, which is capable of using MTBE as its sole carbon and energy source at relatively rapid rates. PM1 is a Gram-negative rod and, based on 16S rDNA sequence similarity, is a member of the beta subgroup of Proteobacteria, in the family Comamonodaceae, and closely related to *Aquabacterium*, *Rubrivivax*, *Leptothrix*, *Ideonella*, and *Hydrogenophaga* (Bruns et al., 2001). PM1 rapidly mineralizes MTBE at concentrations up to 500 mg/L in laboratory cultures (Hanson et al., 1999, Deeb et al., 2000) and degrades MTBE when inoculated into groundwater or soil microcosms. The overall goal of our research was to evaluate whether bioremediation with Strain PM1 is feasible for treatment of MTBE-contaminated groundwater.

Objectives

The initial primary objectives of the proposed study were to: 1) measure the potential for and rates of biodegradation of MTBE in vadose and groundwater materials (contaminated and non-contaminated) inoculated with Strain PM 1, and 2) measure

the survival of Strain PM 1 in such samples. After writing the proposal, we were given an opportunity at Port Hueneme Naval Base to conduct a pilot field study of MTBE bioremediation using Strain PM1. Thus our major objectives were carried out in the field, rather than in laboratory microcosms as originally proposed. In addition, we placed more emphasis on determining the efficacy of in situ bioremediation using Strain PM1 rather than on basic characterization of PM1.

Experimental Procedures

Routine growth and maintenance of Strain PM 1

Cultures were grown in mineral salts medium (MSM) with MTBE as the sole carbon and energy source. Cultures were incubated in 250-ml bottles sealed with teflon-lined Mini-Nert caps at 25°C in the dark on an orbital shaker. After growth, cultures were centrifuged, the cell pellet washed twice and resuspended, and then used in inoculation. Inoculation densities were determined spectrophotometrically based on standard curve OD_{550} measurement vs. cell plate counts.

Measurement of MTBE biodegradation potential in environmental samples

A series of laboratory studies were conducted to examine the ability of strain PM1 to degrade MTBE in environmental samples. Groundwater core samples were inoculated with MTBE-grown Strain PM 1, and MTBE degradation was measured by gas chromatography of 50 ul headspace samples using a Shimadzu GC-14A equipped with a photonionization detector. Rates of MTBE degradation were calculated based on time course analysis of MTBE disappearance from the microcosms. All experiments were performed in triplicate and inoculated samples were compared to sterile controls.

Port Hueneme field trial

A field trial was initiated in a shallow, anoxic, MTBE-contaminated groundwater aquifer at Port Hueneme Naval Construction Battalion Center in Oxnard, California. Port Hueneme, located along the Central California coast, is the location of one of the nation's longest dissolved plumes of MTBE (Figure 1). A 1,500 m long plume resulted from a large release of gasoline containing MTBE from a Navy NEX gas station beginning in approximately 1985. The UCD test plots (each 2.7 x 1.4 m) are located 610 m down gradient from the source of MTBE, which is a service station on the base (Figure 2). Two small pilot test plots (A and B) located down gradient from an MTBE source were injected, by sparging, with pure oxygen at two depths. One plot (B) was also inoculated with Strain PM1. Plot A and plot B are separated by 13.7 m . Each plot contains five rows of three points, with each point containing two 1.9 cm wells: a shallow well (2.4 to 3.3 m) and deep well (4.9 to 5.8 m). For example, a well in the oxygen and PM1 plot located in the second row, centerline at the deep location would be called B22D (Figure 3). Groundwater in the wells was sampled for MTBE, oxygen, microbial populations and other groundwater characteristics. Oxygen delivery began late October of 1999. Strain PM1 was added to Plot B on November 8, 1999 (density of approximately 10^9 cells per ml in the final injection solution of 220 gallons mineral salt media). Strain PM1 was injected using

a Geoprobe unit at 9 points in the injection bed, within two rows interspaced between the oxygen sparging wells (Figure 3).

Growth of strain PM1 inoculum

Strain PM1 was grown up at facilities at Lawrence Livermore National Laboratory. The cells were grown in a 1500L batch reactor on multiple inputs of 1000 mg/L ethanol. After growth on ethanol, the cells were fed sequentially with 83, 192, and 255 mg/L MTBE. After removal of MTBE, the cells were harvested via continuous centrifugation and stored at 4°C until use. One week prior to injection, the cells were diluted in mineral salts media in four 55 gallon plastic drums, equipped with air sparging units and stirrers attached to a motor. Cells were fed 100 mg/L MTBE on a daily basis for the week and then transported to Port Hueneme. At Port Hueneme, the cells were mixed in a 1:1 ratio with groundwater from the site before injection.

Groundwater sampling protocol

MTBE samples were collected using Cole Parmer Masterflex peristaltic pumps. One well volume of groundwater, 450 ml for deep and 160 ml for shallow wells, was removed prior to sample collection. For MTBE analyses, groundwater samples were placed in 40 ml amber glass vials with septa tops containing 10% sodium azide as a sterilization agent. For DNA analyses groundwater was placed in 250 ml sterile plastic bottles and shipped on ice to the lab in UC Davis. To prevent cross-contamination between different plots, sterile tubing was used for each sampling event and plot.

MTBE Analysis

MTBE was analyzed in 10-ml aliquots using purge-and-trap concentration followed by gas chromatography. Instrumentation included a Tekmar LSC 2000 Purge-and-trap, a VOCARB 3000 Trap, Tekmar 2060 Autosampler and a Shimadzu GC-14A gas chromatograph with a 15-m 0.53-mm DB1 column (J&W Scientific, Folsom, CA) with a photoionization detector. This method provided a detection limit of 5 ug MTBE per L. A five-point calibration curve including 0, 0.5, 1.0, 4.0 and either 2.0 or 10 mg MTBE per L was used for each run. The purge and trap procedure included a standby temperature of 35° C, purge time of 10 minutes, desorb preheat of 160°C, desorb of 4 minutes at 175°C, and bake at 4 minutes at 260°C. The temperature program to reduce peak tailing included an initial temperature of 35°C for 5 minute, temperature increase at a rate of 25°C/min, and final temperature of 150°C for 10 minutes.

DNA extraction from groundwater

DNA was extracted from 5 ml microcosm or 130 ml ground water samples using the same protocol. Bacterial cells were concentrated from water samples on white polycarbonate filters (diameter 47 mm, pore size 0.2 μm; type GTTP 2500; Milipore, Germany) placed on nitrocellulose support filters (47 mm, 0.45 μm) by applying a vacuum. After freezing the tubes in liquid nitrogen the filters were broken into small pieces, 750 μl Ground Water Extraction Buffer (10 mM Tris-HCl, 1 mM EDTA pH 7.8, 0.2 % SDS) and 0.25 g glass beads were added to each tube. After bead beating

(speed 4.0, time 20 sec, Savant Instrument, Bio 101) the tubes were placed in a boiling water bath for 1 minute. The samples were then cooled on ice and centrifuged for 2 min at 12,000 rpm. A 0.4 volume of 7.5 M ammonium acetate was added to the supernatant. Crude lysates were extracted two times with chloroform: isoamyl alchohol (24:1). The nucleic acids from the aqueous phase were concentrated and washed with TE (10 mM Tris-HCl, 1 mM EDTA pH 7.8) in a microconcentrator (Centricon 100, Amicon), and the preparations were reduced to a final volume of 30 µl.

ITS fingerprinting

The DNA-based approach for community fingerprinting involved analysis of the intergenic transcribed spacer (ITS) region of the microbial chromosome. In this technique, primers specific for the highly conserved regions at the 3' end of the 16S rDNA gene and the 5' start of the 23S rDNA gene are used for PCR amplification of the intervening less conserved region. This method is successful in distinguishing closely related bacterial strains, and thus provides a high level of discrimination for use in tracking specific bacterial populations in environmental samples. To generate ITS patterns, purified DNA from mixed and pure cultures was amplified by PCR using the primers 1406f (5'-TGYACACACCGCCCGT-3') and 115r (5'-GGGTTBCCCCATTCRG-3') (Borneman and Triplett, 1997). The 1406f primer is complementary to positions 1391-1406 of the *E. coli* small subunit rRNA gene, and the 115r primer corresponds to positions 115-130 of the *E. coli* large subunit rRNA gene. These primers amplify DNA fragments ranging from 140 to 1500 bases in length, depending on the ITS regions found within bacterial DNAs. PCR products were then applied to wells in 5% polyacrylamide gels and subjected to electrophoresis. Gels were stained with 0.01% SYBR Green I stain illuminated with UV light, and photographed through a yellow filter with a charge-coupled-device camera.

TaqMan assay and quantitation.

We developed a quantitative real-time TaqMan PCR method for detection of 16S rDNA sequences specific to the MTBE-degrading strain PM1 (Hristova et al., 2001). The TaqMan method uses a fluorescent oligonucleotide probe with a 5' reporter dye and 3' quencher dye. During the PCR, the 5'-3' -nuclease activity of *Taq* DNA polymerase cleaves nucleotides from an oligonucleotide probe annealed to a target DNA strand. As the amplification reaction proceeds, more amplicons become available for probe binding, and consequently, the fluorescence signal intensity per cycle increases (Heid et al., 1996). The initial copy number is estimated from the exponential phase of product accumulation and by comparison to a standard curve. PCR was performed in 25-µl volumes using MicroAmp Optical 96-well reaction plates and MicroAmp Optical Caps (Applied Biosystems). A 113 bp product was amplified using primers 963F and 1076R and probe 1030T (Hristova et al., 2001). DNA extraction was performed in duplicate or triplicate and two PCR reactions were run for each extraction. Dilution series were tested in triplicate.

A 5 µl of diluted template was added to 20 µl PCR master mix [12.5 µl of TaqMan Universal Master Mix (a 2x-concentrated mixture of AmpliTaq Gold™ DNA Polymerase, uracyl-N-glycosylase (UNG), dNTPs with UTP, passive reference dye and optimized buffer)], 2.5 µl mixture of 100 nM oligonucleotide primers and 100

nM TaqMan probe and 6 µl ddH_20. All reagents were obtained from Applied Biosystems. After an initial incubation at 50°C for 2 min to activate the UNG and a denaturation phase of 10 min at 95°C, the temperature profile followed a two-step cycle pattern with a combined annealing and primer extension phase at 60°C for 1 min, and a short denaturation at 95°C for 15 sec. Forty cycles of amplification, data acquisition and data analysis were carried out routinely in an ABI Prism 7700 Sequence Detector (PE, Applied Biosystems).

DGGE (denaturing gradient gel electrophoresis) separates double-stranded DNA fragments of the same size, based on properties defined by the genetic sequence and secondary structure of the fragments in an increasing gradient of chemical denaturant (urea plus formamide). Universal bacterial DGGE primers (Muyzer et al., 1993;1998) has been used to detect and identify dominant species, which constitute at least 1% from the total microbial community. Primers specific for PM1 16S rDNA sequences have been designed in our lab (Hristova and Scow, unpublished) and used for DGGE analysis and sequencing of strain PM1 and PM1-like organisms in the groundwater.

Data Analysis and Interpretation

TaqMan PCR data were analyzed with Sequence Detector Software (SDS, Version 1.7). Threshold determinations are automatically performed by the instrument for each reaction. The threshold is defined as 10 times the standard deviation of the normalized fluorescent emission of the non-template control reaction. The cycle at which a sample crosses the threshold (C_T, a PCR cycle where the fluorescence emission exceeds that of non-template controls) is called threshold cycle C_T. The C_T values were exported into Microsoft Excel for further statistical analysis. Three PCR reactions were performed for each environmental DNA extraction. For ITS fingerprints a band-matching algorithm was used to calculate pair-wise similarity matrices with the Dice coefficient and/or densitometric curve based algorithm using the Pearson correlation coefficient (GelCompare software, version 4.0, Applied Maths, Belgium). Cluster analyses of similarity matrices were performed by the unweighted pair group method using arithmetic averages (UPGMA).

Results

Microcosm bioaugmentation potential studies

Addition of Strain PM1 to groundwater sediments from Port Hueneme spiked with 10 mg MTBE per L resulted in more rapid rates of MTBE removal than what was measured in uninoculated sediments (Fig. 4). After degradation of the initial input of MTBE within 5 days in the inoculated flasks, MTBE was added again, multiple times, at the same concentration. These later inputs of MTBE were degraded more rapidly than was the first input of MTBE, suggesting an increase in the initial population density of MTBE-degrading organisms. The uninoculated samples also degraded MTBE, although after a longer lag period (200 h, with complete

degradation after 460 h) than what was observed in the inoculated flasks. Subsequent additions of MTBE, however, were degraded in the uninoculated sediments at rates similar to those measured in the inoculated flasks.

MTBE removal at Port Hueneme

Oxygen delivery to plots A and B was initiated in late October of 1999; intensive sampling of dissolved oxygen in groundwater was conducted to determine when sufficient oxygen was present to support the activity of PM1. Modifications had to be made to the original design of the wells to increase oxygen delivery to locations where much of the MTBE was present. By early November, high concentrations of dissolved oxygen were measurable in almost all of the shallow wells and in some, but not all, of the deeper wells at the site. Strain PM1 was added to the plot on November 8, 1999. We conducted frequent sampling (approximately every month) for oxygen and MTBE and, less frequently, microbiological analyses in the center well of each row (Fig. 3). Field monitoring of MTBE began with the injection of stain PM1 and is referred to as time zero in the presentation of results.

Figs. 6a and 6b show MTBE removal along the distance from the injection bed in the shallow centerline of plot B and plot A, respectively. The initial MTBE concentrations in the shallow zone ranged from 2.5 – 3.5 mg/L in plot B, and were much lower in plot A (below 0.14 mg/L, 2 m downstream). In the down gradient wells and immediately upstream near the oxygen release wells, MTBE concentrations decreased substantially in the shallow zone of the groundwater in both plots, even in the absence of Strain PM1 (plot A). After six months of treatment MTBE concentrations declined to 0.008 mg/L or non-detect (<0.005 mg/L) in plot A and to 0.09 mg/L or non-detect in Plot B.

In the deeper zone, initial MTBE concentrations ranged from 5 mg/L upstream to < 1 mg/L downstream in Plot A and ranged from 5.7 – 9.3 mg/L in plot B. The differences in concentrations between Plots A and B reflected spatial variability in MTBE concentration as well as evidence that some treatment had already begun one month after oxygen injection began (=T0). Downstream MTBE concentrations decreased substantially in plot A to below 0.11 mg/L (Fig. 7a) but only slightly in plot B (Fig. 7b). Difficulties in delivery of oxygen to the deep zone in plot B, as evidenced by the low dissolved oxygen concentrations present, was likely responsible for low rates of MTBE removal at that location (Fig. 8 b). Well pump tests indicated that groundwater flow was substantially slower in the shallow than deep zones, and slower in plot B than A.

PM1 detection and quantification in Port Hueneme ground water

Method development. A TaqMan real time quantitative PCR assay was developed in our lab to quantify Strain PM1 in laboratory and field samples (Hristova et al., 2001). Microcosm studies were conducted to determine the relationship between PM1 cell density (by Taqman PCR) and aerobic MTBE biodegradation in mineral media, inoculated groundwater, and in groundwater collected from the bioaugmentation study at Port Hueneme. Ten ppm MTBE (Fig. 5A) was biodegraded by ~$1x10^6$

CFU/ml PM1 in mineral media to undetectable levels within 250 h after a short lag period. Strain PM1 inoculated into groundwater samples collected from locations upgradient of the field site (e.g., samples in which PM1 was not initially present) also rapidly degraded MTBE (Fig. 5B). A 2 and 1.5 log increase in PM1 cell density was observed in groundwater and mineral media microcosms, respectively (Fig. 5A and B) and increases in cell density were concomitant with declines in MTBE concentration. This relationship supported the previous observation (based on cell protein, Hanson et al., 1999) that MTBE can support growth of PM1. Total heterotrophic plate counts performed at the beginning and end of the experiment also showed a 2 and 1.5 log increase in cell densities in the inoculated groundwater and mineral media, respectively.

Groundwater samples were also collected from the field test plot at Port Hueneme (well B32D) seven months after injection of strain PM1 (Smith, 2000). At the time of sampling, the density of PM1 was $3.3\pm2.4 \times 10^4$ per ml as detected by TaqMan assay. Following incubation with added MTBE, a one log order increase in PM1 density was measured by both the TaqMan assay (Fig. 5C) and plate counts (data not shown). The rate of MTBE biodegradation was lower (8.5 ppm removed in 500h) than what was observed in the other microcosms, probably due to the presence of densities of PM1 much lower in the samples collected from the field plots than in the samples inoculated in the lab.

Application of 3 nucleic acid-based methods for detection of Strain PM1 in field samples. Three sets of PM1 specific primers (16S and ITS rDNA) were used to detect PM1 in Port Hueneme samples. Using ITS specific primers of DNA extracted from samples collected 24 days after injection of PM1, we detected PCR products with the expected length of 444 base pairs in all samples from plot B and plot A with 3 exceptions (wells B-12-D, B-52-D and A-42-S, Fig. 9).

Using TaqMan PCR, we quantified the density of PM1 cells in samples (shallow and deep) from plots B and A (Fig. 10a and b) 24 days after injection (T1) of PM1. PM1 was detectable at densities ranging from 10^2 to 10^5 per ml groundwater. Higher densities of PM1 were detected in deep than shallow depths of both test plots. Up to one log order higher PM1 cell densities were quantified in plot B than plot A. Cell densities declined substantially over the course of the field trial (T4, T7, Fig. 10) in both the shallow and deep zones. The specificity of the TaqMan PCR primers for Strain PM1 was confirmed by sequencing the PCR products. Sequence analyses of 16S rDNA TaqMan PCR products obtained with extracted DNA from plots B and A showed 99-100% similarity with the bacterial strain PM1 with 2 exceptions (A32S – T1 and A22D -T4 <99%, data not shown). There was a strong correspondence between positive ITS PCR detection of strain PM1 and TaqMan detection of PM1 in samples containing more than 10^3 cells per ml. At lower densities, the ITS method was not sensitive enough to detect Strain PM1.

A third set of PM1 specific primers were designed to target a different region of 16S rRNA molecule than that targeted by the TaqMan primers. Using the second set of 16S primers with DGGE analysis provided additional evidence of the presence of PM1 in both plots A and B. DNA sequences from the 375 bp band (from plot A, and

plot B, Fig. 11 a) were 97-100% similar to the sequence of PM1's 16S rDNA. Fig. 11 b) depicts the results of the DGGE analysis with universal bacterial primers. Bands in the groundwater DNA corresponding to the band associated with a pure culture of PM1 are indicated by arrows.

In conclusion, our results suggest the presence of a naturally occurring Strain PM1 at Port Hueneme. We could not definitively determine, however, whether the presence of PM1 in plot A was due to movement from the inoculated plot (by sparging) or if it was native to the site. We also tested whether PM1 was present in locations far removed from the field site. Eight groundwater samples were collected outside of the field-tested plots 7 months after injection of strain PM1 – 2 of them close to our plots, 3 upstream and 3 downstream of the plots (Table 1). DNA was extracted and tested for PM1 presence by ITS and TaqMan PCR. PM1 was detected by both PCR techniques in only one of the samples located far away (downstream) from the injection bed (CBC-51). Two more samples – one close to plot B (CBC-60-CS) and one further downstream (PHB4-1) showed PM1 presence by TaqMan real time PCR.

We isolated a bacterial strain from Port Hueneme site PHB4 (623 m downstream from our field site), where 5.7×10^3 CFU/ml PM1 are present in the groundwater as estimated by TaqMan PCR (Table 1). Sequencing of portions of the genome of this bacterial strain indicated that the new isolate was 100% similar to PM1 in the 16S rDNA (Fig. 11 b) and 99% in the ITS region. The isolate was able to degrade MTBE in laboratory microcosms but at rates far slower than measured for Strain PM1 (data not shown).

ITS fingerprinting of microbial community in contaminated groundwater

We amplified groundwater DNA by ITS using primers common to all bacteria to measure changes in the community in response to MTBE biodegradation and bioaugmentation activities (Fig. 12). The largest differences in the microbial community profiles were between the shallow and deep groundwater samples (at most 15% similar with the sole exception of well A42) regardless of whether they were from plot A or B, as evidenced by the two major cluster separation (Fig. 12). Within the shallow cluster, the plot B samples were 69% similar to one another and in plot A only 40 to 60 % similar one another. Microbial communities in the plot A and plot B deep wells were 74–84% similar. At the same time, samples from wells upstream of the oxygen release (A12D, B13D) and far downstream (B52D) were only 38-68% similar to the samples within the treatment bed. These results suggest that differences with depth in groundwater characteristics and oxygen delivery had a larger influence on microbial community composition than did the fact that Strain PM1 was injected directly into the plot (i.e., plot B). We also cannot rule out that the lack of difference between the two plots may be due to displacement of PM1 into plot A.

Conclusions

The overall significance of our study is that bioremediation, both through inoculation or by stimulating native organisms, shows promise as a technology for cleaning up MTBE-contaminated groundwater. A major objective of our study was to determine if bioaugmentation with Strain PM1 was necessary to achieve MTBE removal. Our results indicate that both inoculated and uninoculated plots showed similar levels of MTBE removal. Strain PM1 or a naturally occurring PM1-like organism may be responsible for some (or all) of the biological removal of MTBE in the plots as evidenced by molecular detection of Strain PM1 sequences in all samples. Results of controlled microcosm studies provide further evidence that oxygen additions to uninoculated Port Hueneme aquifer sediments stimulate native MTBE-degrading organisms already present in these samples. Once the native community is adapted to MTBE, additional inputs of MTBE are degraded at rates similar to those measured for Strain PM1.

To scale up and apply this technology at different sites will require improvements in the oxygen delivery system and optimization of the needs of the microorganisms in the field. Future bioaugmentation and/or biostimulation field trials using sparging should carefully determine sparging rates that provide enough oxygen to create the aerobic conditions necessary to stimulate biodegradation without adding so much oxygen that i) volatilization becomes the dominant mechanism of contaminant removal or ii) the flow of groundwater is impeded through the treatment bed. Other methods of oxygen delivery may provide oxygen without the disadvantages associated with sparging. An example of an alternative oxygen supply method is the approach of MacKay et al. (2000, 2001), who employ a gentle release of oxygen via diffusion across a semipermeable tubing in response to the aquifer's demand for oxygen. Future work will explore the potential for biostimulation of native degraders within other MTBE plumes

We succeeded in developing a quantitative real time TaqMan PCR assay targeting the 16S rDNA for detection and quantification of the MTBE degrading strain PM1. The method was successful in detecting PM1 in laboratory cultures and inoculated environmental samples (groundwater and sediment), as well as in detection PM1 in the field. Detection of PM1 in groundwater samples at locations outside the field plots also suggested that PM1-like organisms are naturally occurring in MTBE-contaminated groundwater at Port Hueneme. Future effort will be directed at determining how widespread is the native PM1 organism in the environment and on linking MTBE biodegradation rates to the presence of native PM1.

Contributions of the project

Publications:

Hristova, K. R., C. M. Lutenegger, and K. M. Scow. 2001. Detection and Quantification of MTBE-degrading Strain PM1 by Real-Time TaqMan PCR. Appl. Environ. Microbiol. (in press).

Schwartz, E., and K.M. Scow. 2001. Repeated inoculation as a strategy for the remediation of low concentrations of phenanthrene in soil. Biodegradation (in press).

Bruns, MA; Hanson, JR; Mefford, J; Scow, KM. 2001. Isolate PM1 populations are dominant and novel methyl tert-butyl ether-degrading bacteria in compost biofilter enrichments. Environ. Microbiol. 3:220-225.

Deeb, R.A., H.Y. Hu, J. R. Hanson, K.M. Scow, and L. Alvarez-Cohen. 2000. Substrate interactions in BTEX and MTBE mixtures by an MTBE-degrading isolate. Environ. Sci. Technol. 35:312-317.

Deeb, R.A., K.M. Scow, and L. Alvarez-Cohen. 2000. Aerobic MTBE biodegradation: An examination of past studies, current challenges and future research directions. Biodegradation 11:171-186.

Scow, K.M., E. Schwartz, M.Johnson, and J.L. Macalady. 2000. Measurement of microbial diversity. In: Encylopedia of Biodiversity. (in press).

Scow, K.M. 2000. Soil microbiology. In: Encyclopedia of Microbiology. Academic Press (in press).

Schwartz, E., S.V. Trinh, and K.M. Scow. 2000. Measuring growth of a phenanthrene degrading bacterial inoculum in soil with a quantitative competitive polymerase chain reaction method. FEMS Microb. Ecol. 34:1-7

Macalady, J.L., E. Mack, D. Nelson, and K.M. Scow. 2000. Sediment microbial community structure and mercury methylation in mercury-polluted Clear Lake, CA. Appl. Environ. Microbiol. 66:1479-1488.

Fitzgerald, G., K.M. Scow, and J. Hill. 2000. Fallow season straw and water management effects on methane emissions in California rice. Global Biogeochemical Cycles 14:767-776.

Calderon, F.J., L.E. Jackson, K.M. Scow, and D.E. Rolston. 2000. Microbial responses to simulated tillage in cultivated and uncultivated soils. Soil Biol. Biochem. 32:1547-1559.

El-Farhan, Y., K.M. Scow, and D.E. Rolston. 2000. Kinetics of trichloroethylene cometabolism and toluene biodegradation: model application to soil batch experiments. J. Environ. Qual. 29:778-786.

MacKay, D.M., R. Wilson, G. Durrant, K. Scow, A. Smith, M. Einarson and B. Fowler. 2000. "In Situ Treatment of MTBE by Biostimulation of Native Aerobic Microorganisms". *In*: the Environmental Protection Agency/American Petroleum Institute MTBE Biodegradation Workshop, Cincinnati, Feb. 1-3, 2000.

MacKay, D.M., R. Wilson, G. Durrant, K. Scow, A. Smith, M. Einerson and B. Fowler. 2000 "Field tests of enhanced intrinsic remediation of an MTBE plume". *In*: Abstracts of Papers 219th Meeting of the American Chemical Society, San Francisco, CA. March 26-30th, 2000. 219 (1-2): ENVR 230.

Presentations:

Microbial Ecology of MTBE Biodegradation. Invited talk at the Ecology Graduate Group Odyssey, Tahoe City, CA. 9/18/00.

Bioremediation of MTBE-Contaminated Groundwater. Keynote speaker, Annual Agricultural and Environmental Chemistry Symposium 2000, UC Davis, CA.

Bioremediation of MTBE at Port Heueneme Naval Base. Invited talk at SERDP Advisory Committee Meeting, San Diego, 3/19/01.

Biodegradation of the Fuel Additive, MTBE, in Groundwater. Invited talk at the University of California, Riverside. 5/31/01

Bioremediation of MTBE through Bioaugmentation at Port Hueneme Naval Facility. Invited talk at the Sixth International Symposium of In Situ and On-Site Bioremediation, San Diego, 6/4/01

Molecular Characterization of MTBE-Degrading Isolate, Strain PM1. Poster at the Sixth International Symposium of In Situ and On-Site Bioremediation, San Diego, 6/4/01

Molecular Characterization of MTBE-Degrading Isolate, Strain PM1. Poster at the International Society for Microbial Ecology, Amsterdam, 8/6/01.

Bioremediation of MTBE through Bioaugmentation. Talk at the International Society for Microbial Ecology, Amsterdam, 8/6/01.

References:

Hanson, J.R., Ackerman, C.E. and K.M. Scow. 1999. Biodegradation of Methyl tert-Butyl Ether by a Bacterial Pure Culture. Appl. Environ. Microbiol. 65(11):4788-4792.

Happel, A.M., E.H. Beckenbach and R.U. Halden 1998. An Evaluation of MTBE Impacts to California Groundwater Resources. UCRL-AR-130897: Environmental Protection Department, Environmental Restoration Division, Lawrence Livermore National Laboratory, Livermore, CA. 77pp.

Kirschner, E. M. 1995 Production of top 50 chemicals increased substantially in 1994. Chem.Eng. News. 73:16 - 22.

Squillace, P.J., J.S. Zogorski, W.G. Wilber, and C.V. Price. 1996. Preliminary assessment of the occurrence and possible sources of MTBE in groundwater in the United States, 1993-1994. Environ. Sci. Technol. 30:1721-1730.

Training: This project has provided support (direct funding and indirect) for: 1 postdoctoral fellow (Hristova), 3 graduate students at UCD (Smith, Smith, Gandhi) and 6 undergraduate students (Watanabe, Scott, Smith, Adamson, Lu, Sugitani).

Collaborative Efforts: A number of collaborations have been supported or initiated by this project. My lab continues to collaborate with Doug Mackay from U. of Waterloo, Canada, in a biostimulation study at Vandenburg Air Force Base (Lompoc, CA). Prof Mackay also provides advice on hydrological issues at Port Hueneme. We are also collaborating with Prof. Mike Hyman at NC State on MTBE metabolism, with Prof. Barbara Sherwood-Loller at U. of Toronto on the uses of stable isotopes in detecting MTBE degradation in the field, and collaborated with Rula Deeb (in Prof. Lisa Alvarez-Cohen's lab at UC Berkeley) on BTEX interactions with MTBE biodegradation.

Figure 1. MTBE Groundwater Plume at Port Hueneme

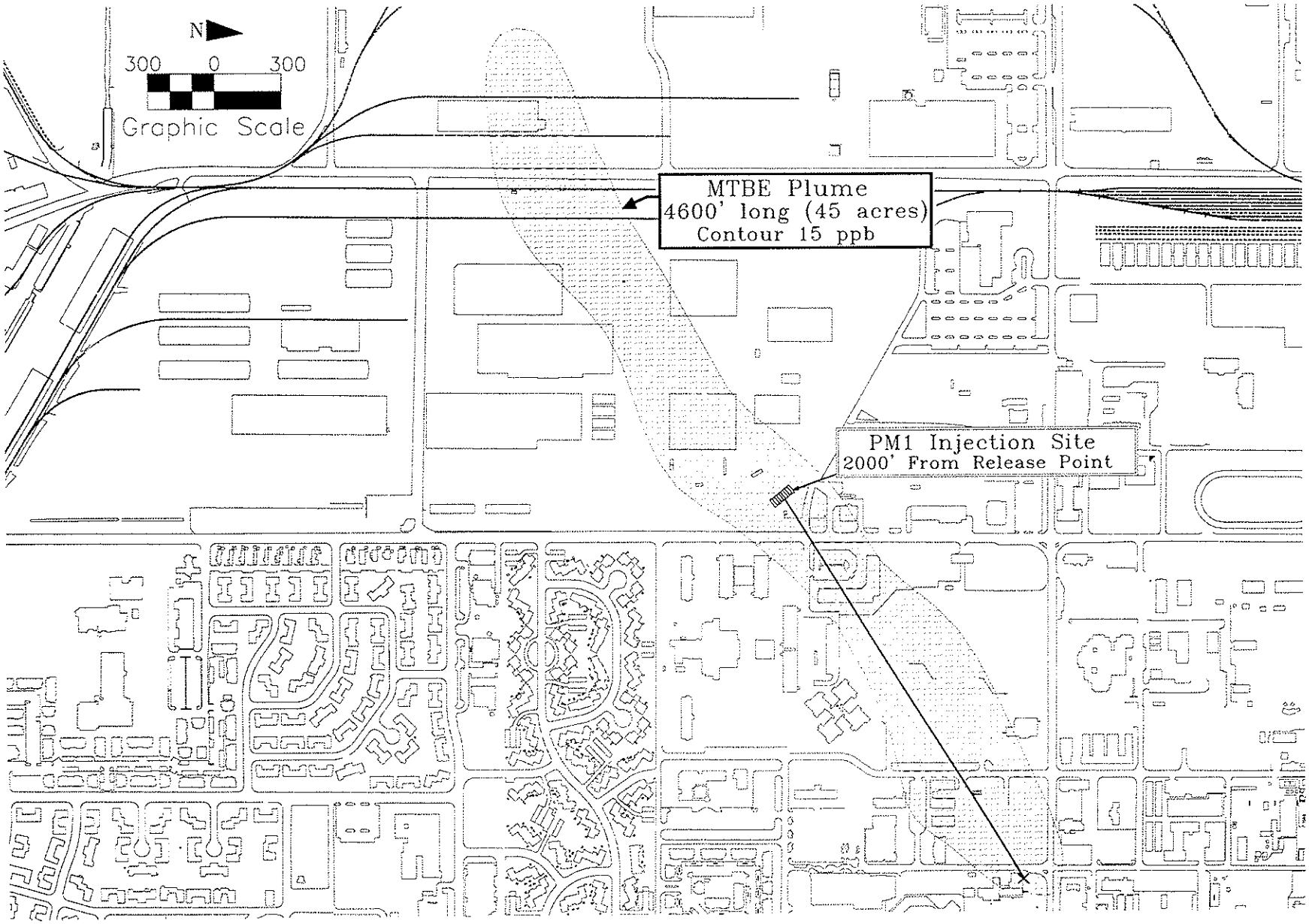
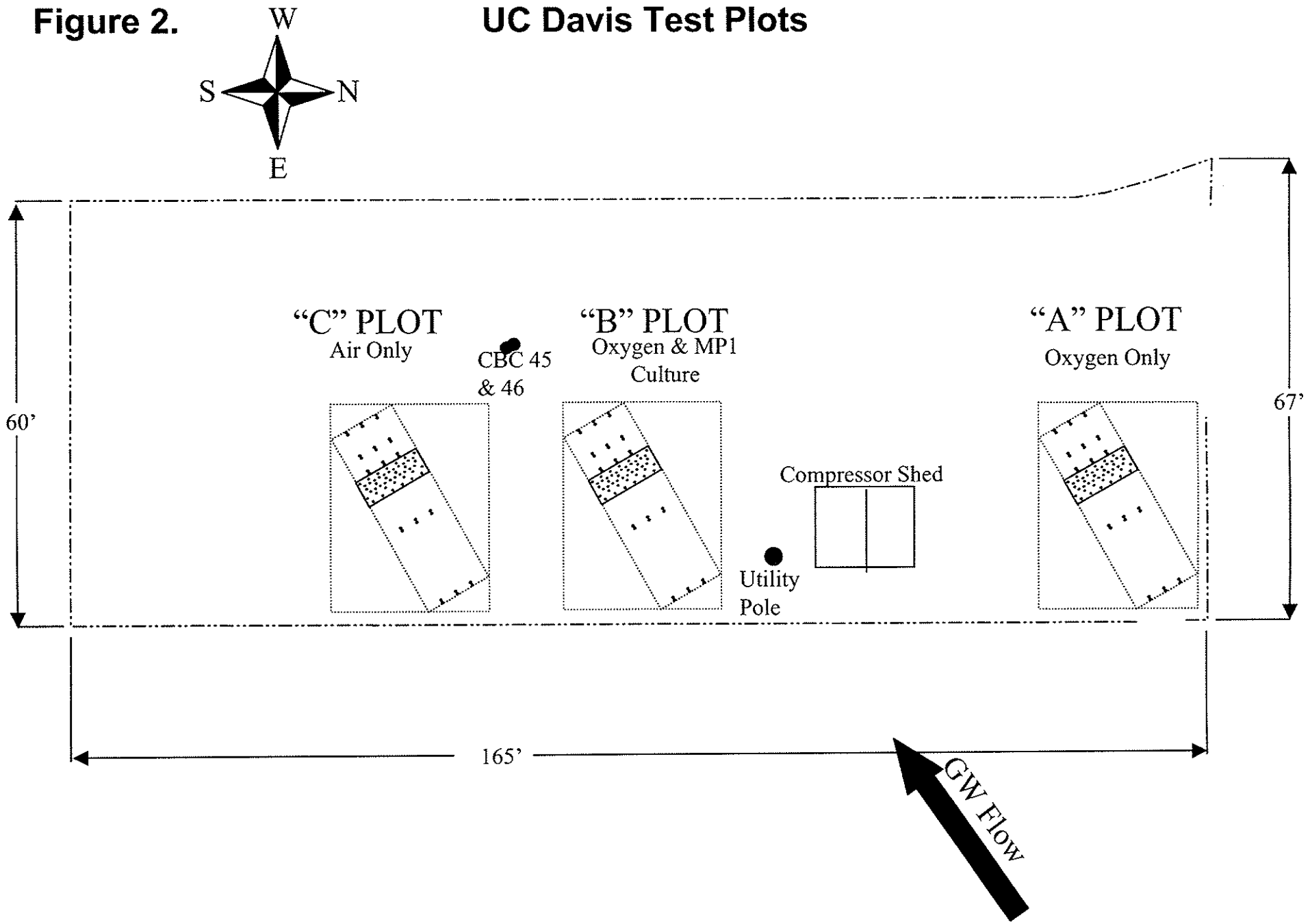


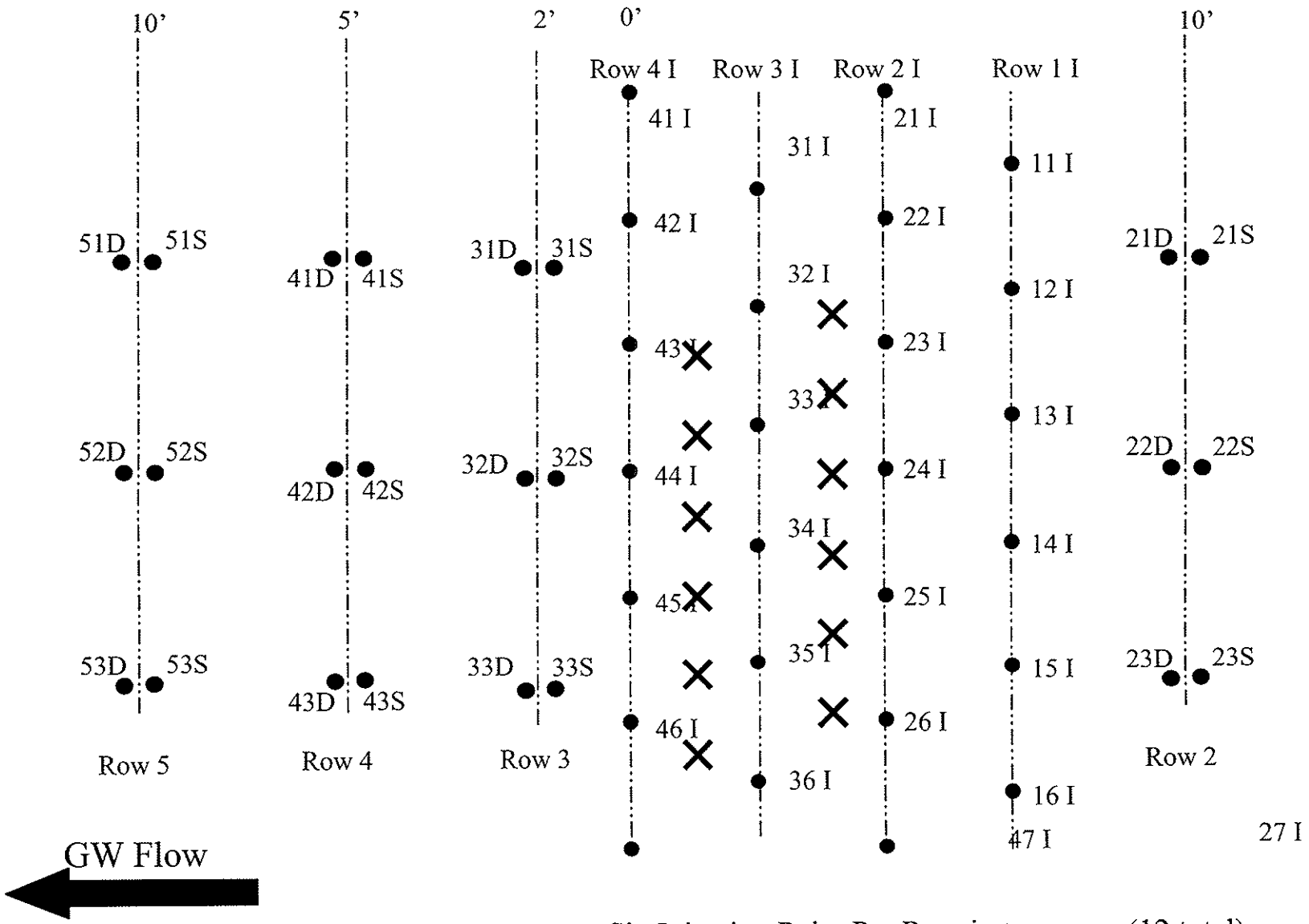
Figure 2. **UC Davis Test Plots**



Installation_layout_UCD.ppt

Figure 3.

Plot B Expanded Well Lay-Out for Well ID



Six Injection Point Per Row in two rows (12 total)

Each injection from 19' to 15', continuos injection measured in 1' increments

Figure 4.

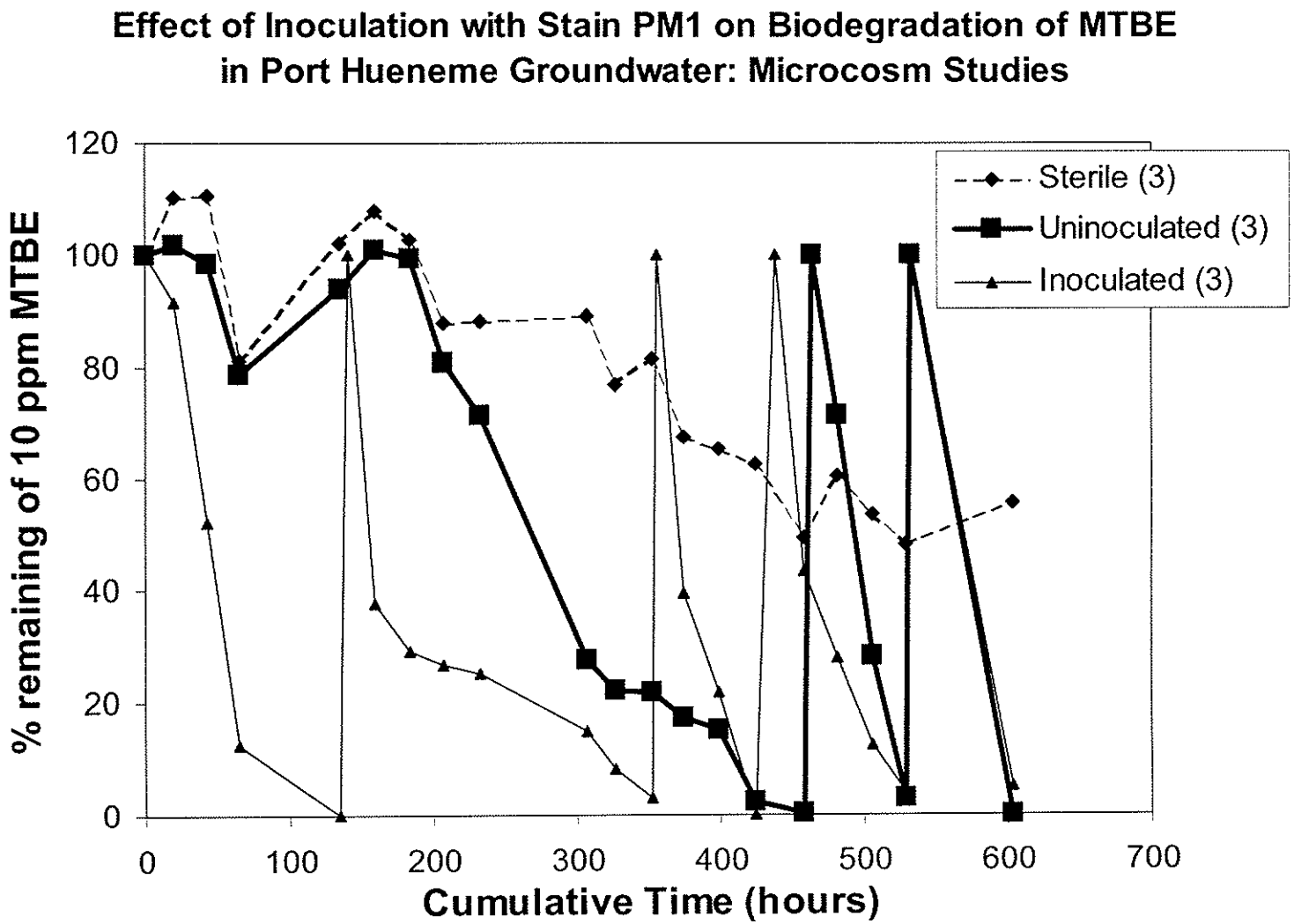
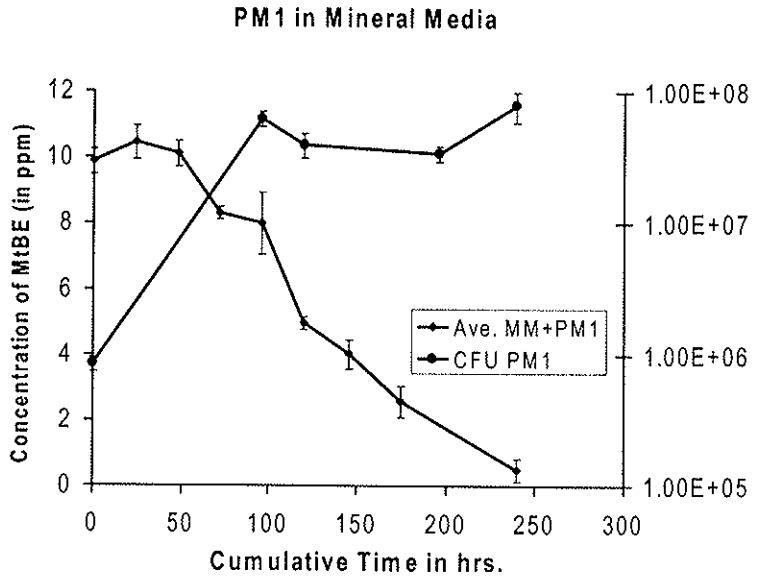
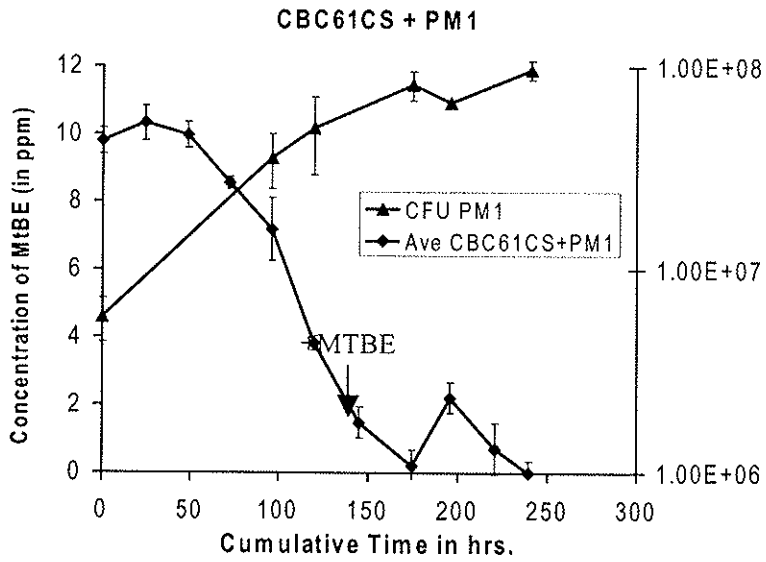

Effect of Inoculation with Stain PM1 on Biodegradation of MTBE
in Port Hueneme Groundwater: Microcosm Studies
% remaining of 10 ppm MTBE
120
100
80
60
40
20
0
0
100
200
300
400
500
600
700
Cumulative Time (hours)
Sterile (3)
Uninoculated (3)
Inoculated (3)

Figure 5. TaqMan estimated PM1 cell density and aerobic MTBE biodegradation

a) Mineral media



b) Inoculated groundwater



c) Groundwater collected from the bioaugmentation study at Port Hueneme

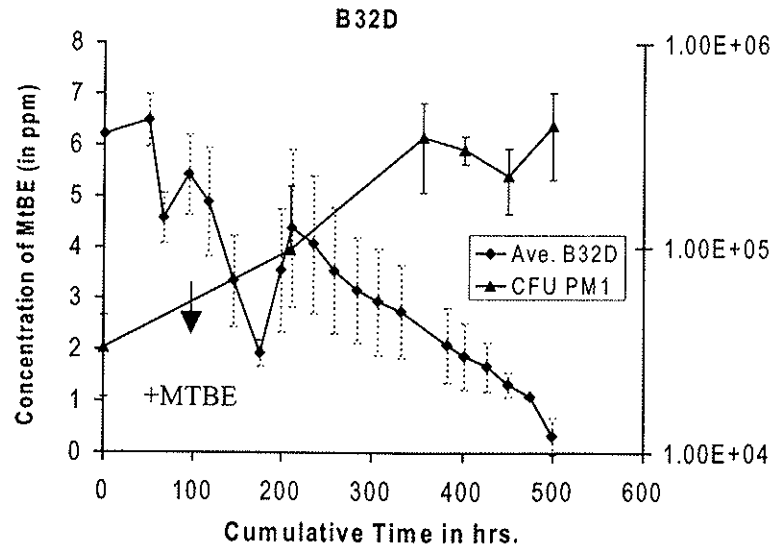


Figure 6 a)

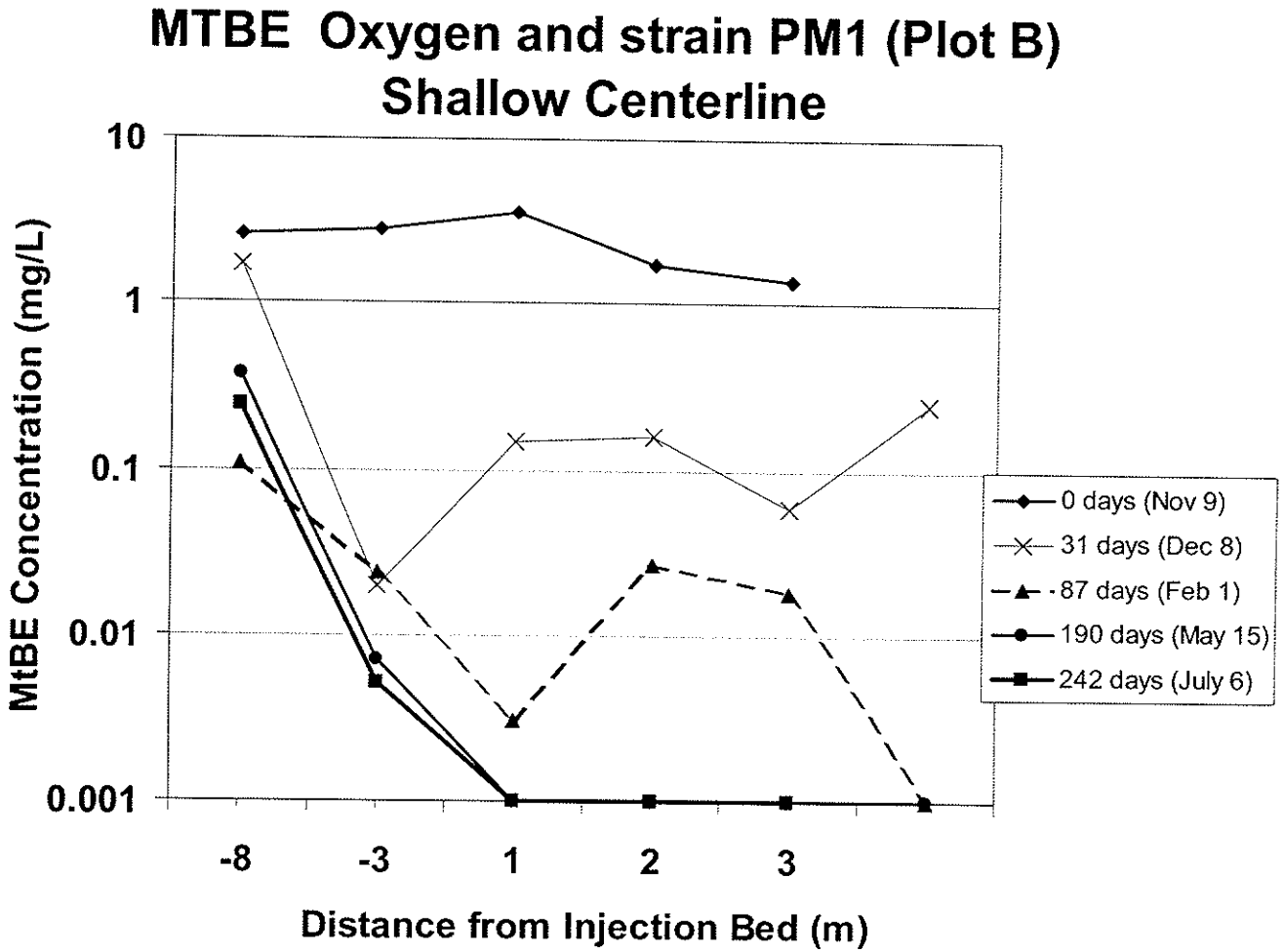

MTBE Oxygen and strain PM1 (Plot B)
Shallow Centerline
MtBE Concentration (mg/L)
10
1
0.1
0.01
0.001
-8
-3
1
2
3
Distance from Injection Bed (m)
0 days (Nov 9)
31 days (Dec 8)
87 days (Feb 1)
190 days (May 15)
242 days (July 6)

Figure 6 b)

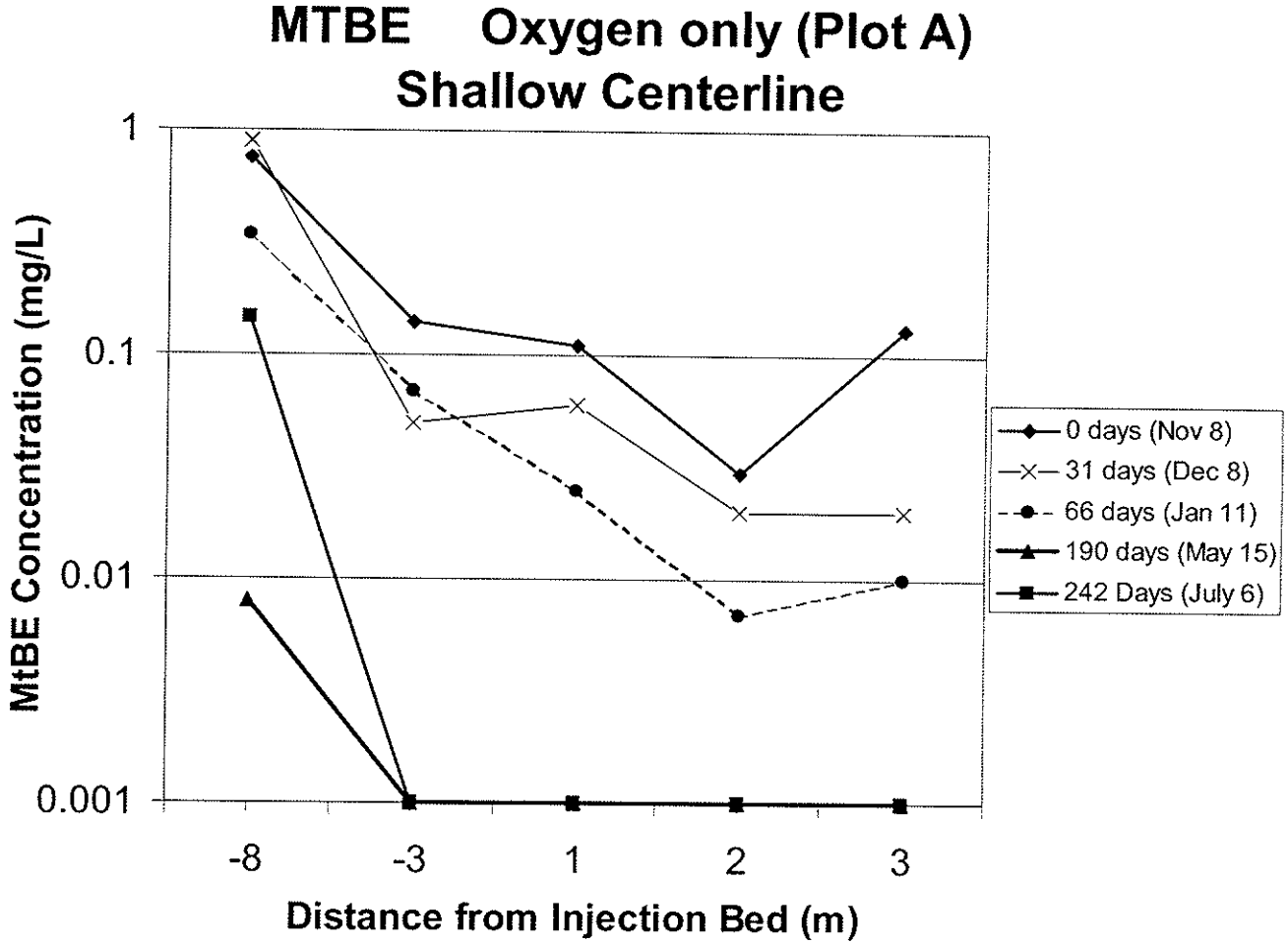

MTBE Oxygen only (Plot A)
Shallow Centerline
MtBE Concentration (mg/L)
1
0.1
0.01
0.001
-8
-3
1
2
3
Distance from Injection Bed (m)
0 days (Nov 8)
31 days (Dec 8)
66 days (Jan 11)
190 days (May 15)
242 Days (July 6)

Figure 7 a)

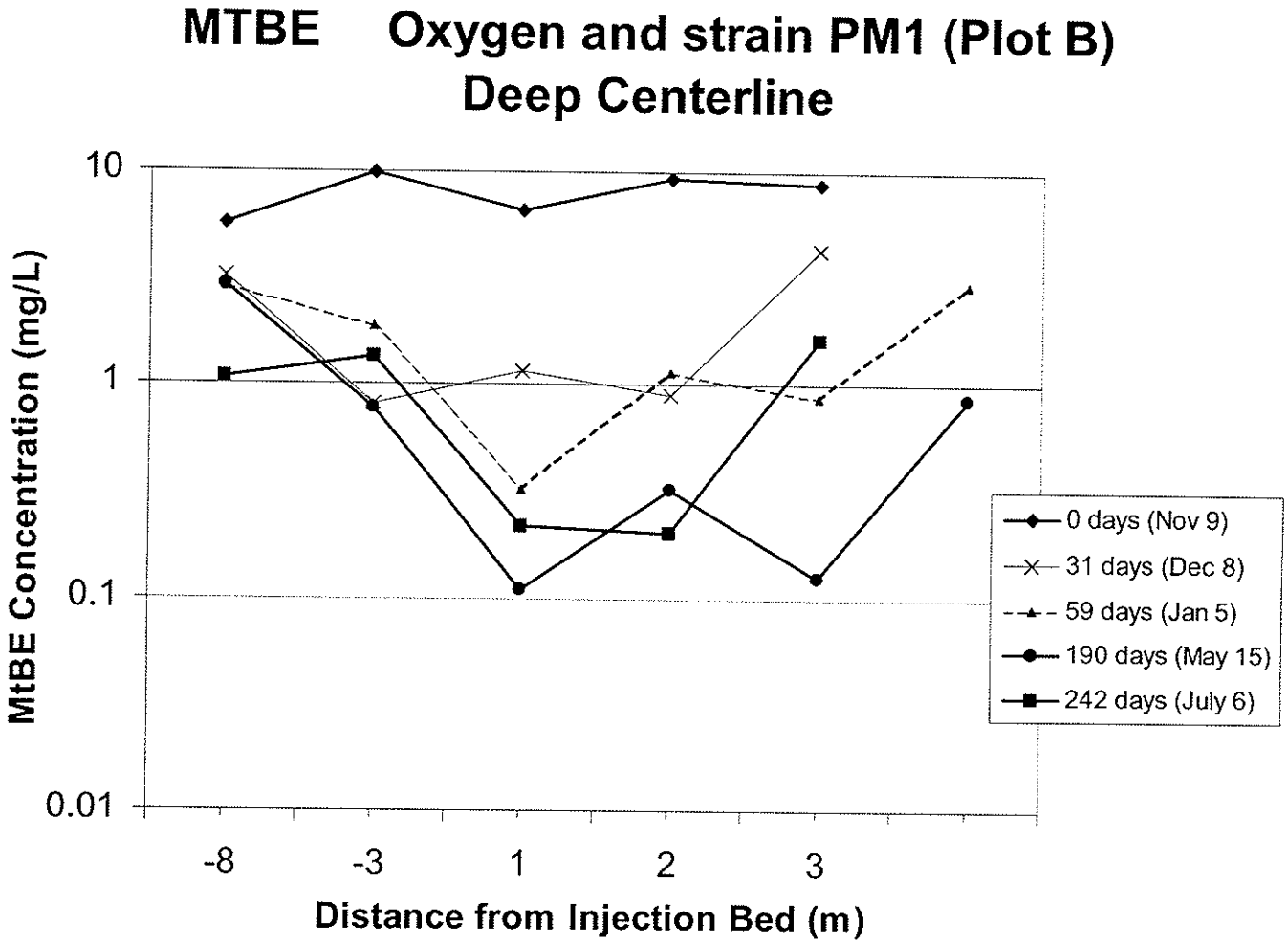

MTBE Oxygen and strain PM1 (Plot B)
Deep Centerline
MtBE Concentration (mg/L)
10
1
0.1
0.01
-8
-3
1
2
3
Distance from Injection Bed (m)
0 days (Nov 9)
31 days (Dec 8)
59 days (Jan 5)
190 days (May 15)
242 days (July 6)

Figure 7 b)

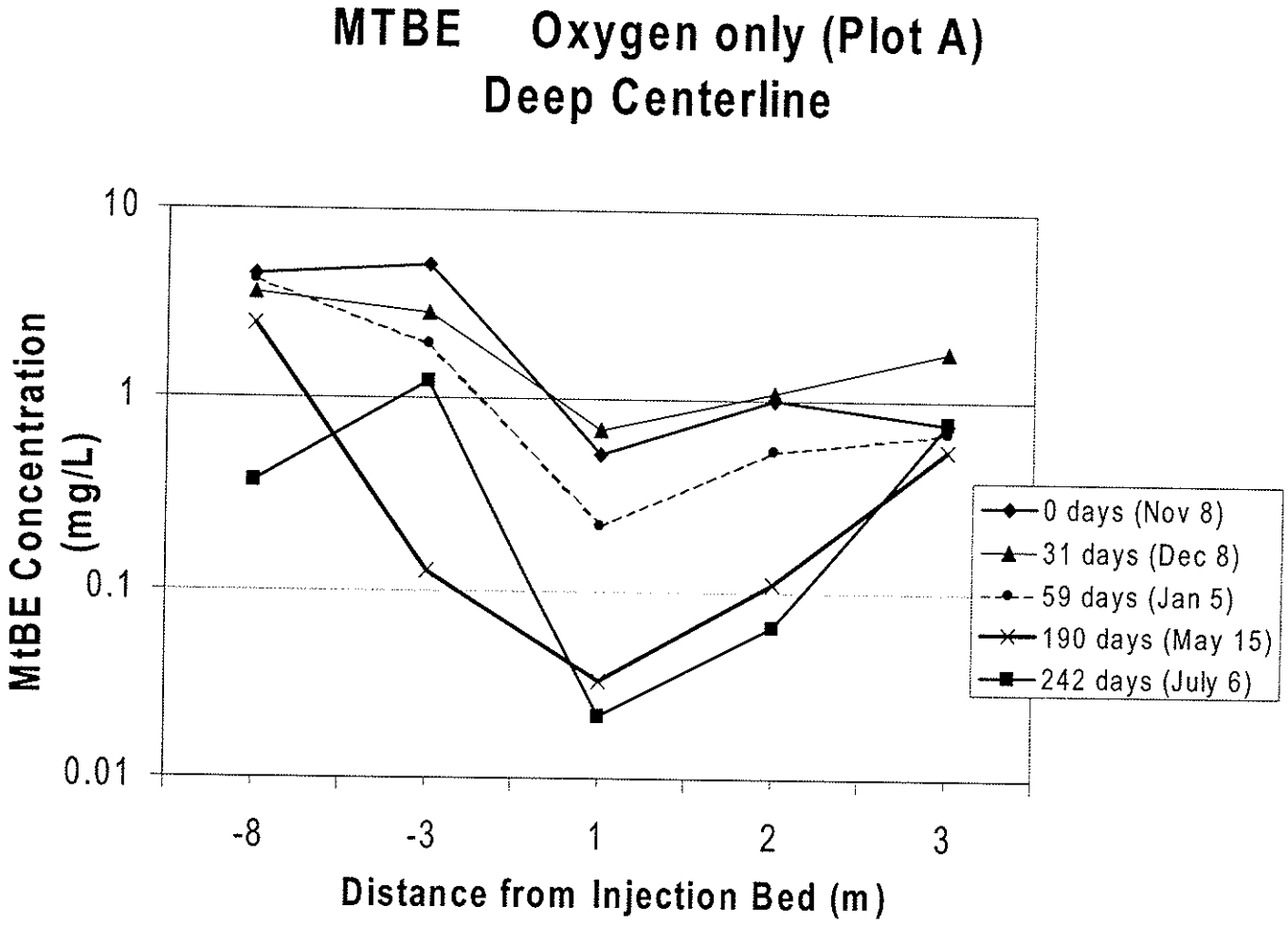

MTBE Oxygen only (Plot A)
Deep Centerline
MtBE Concentration (mg/L)
10
1
0.1
0.01
-8
-3
1
2
3
Distance from Injection Bed (m)
0 days (Nov 8)
31 days (Dec 8)
59 days (Jan 5)
190 days (May 15)
242 days (July 6)

Figure 8 a).

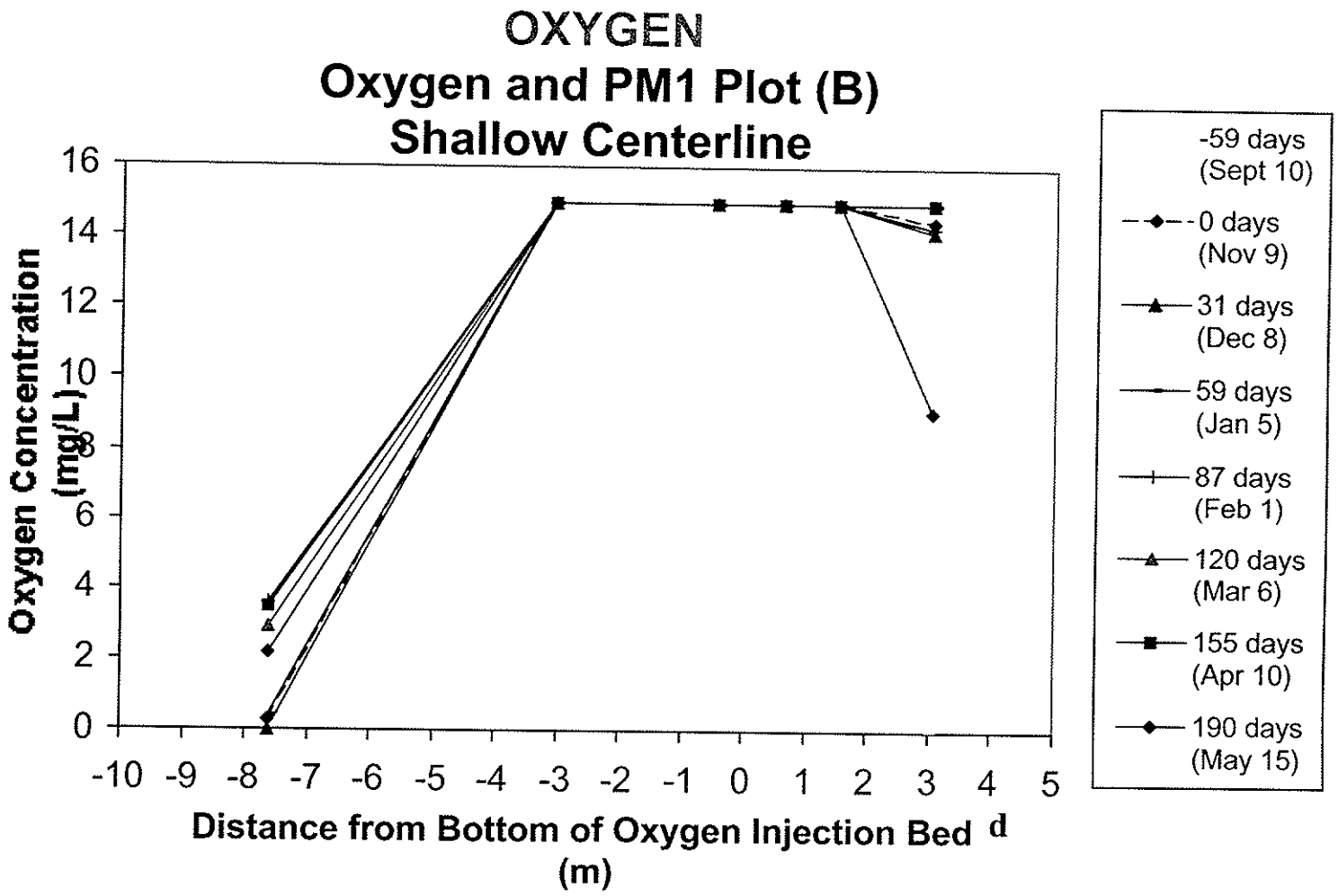

OXYGEN
Oxygen and PM1 Plot (B)
Shallow Centerline
Oxygen Concentration (mg/L)
16
14
12
10
8
6
4
2
0
-10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5
Distance from Bottom of Oxygen Injection Bed (m)
d
-59 days (Sept 10)
0 days (Nov 9)
31 days (Dec 8)
59 days (Jan 5)
87 days (Feb 1)
120 days (Mar 6)
155 days (Apr 10)
190 days (May 15)

Figure 8 b).

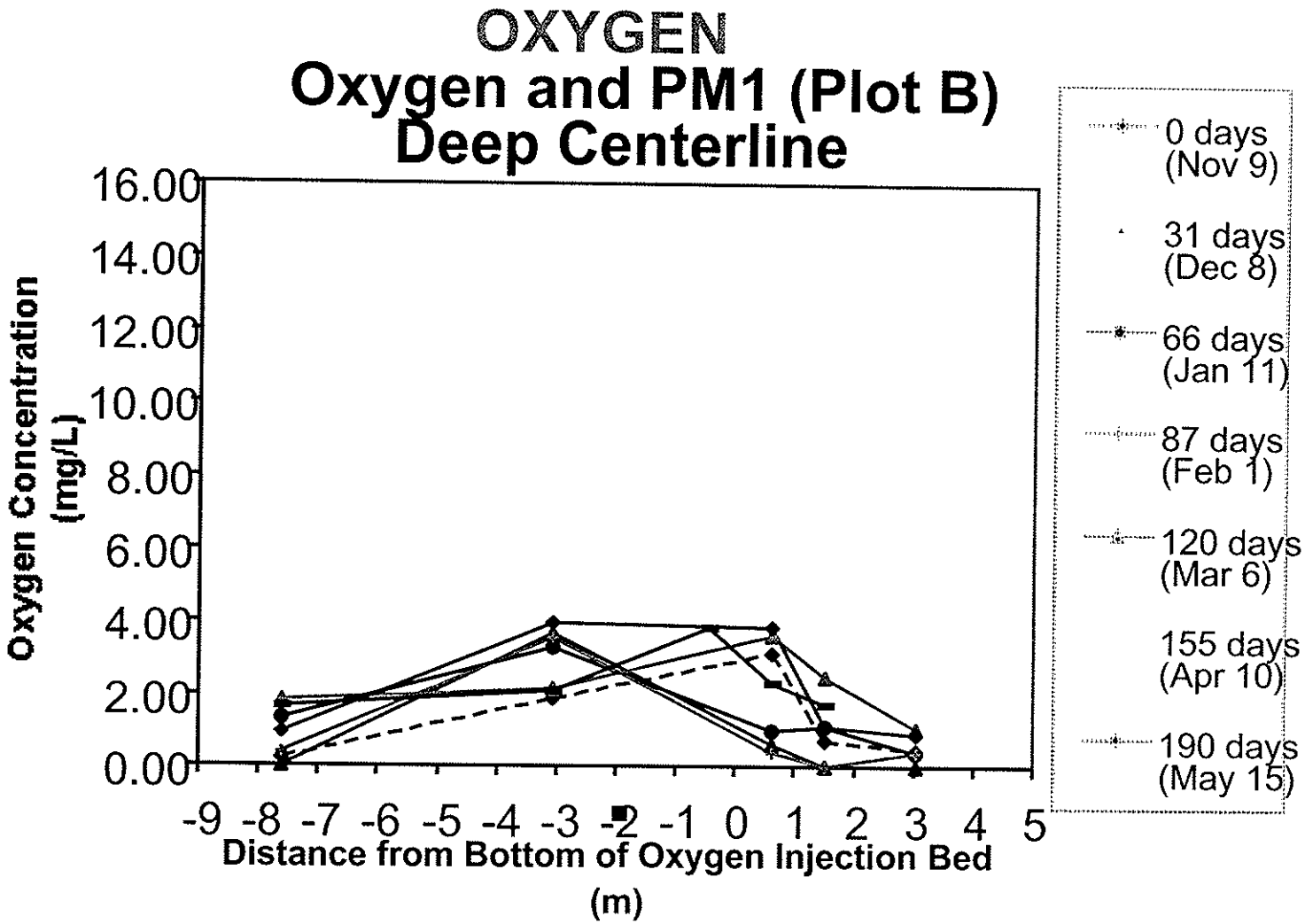
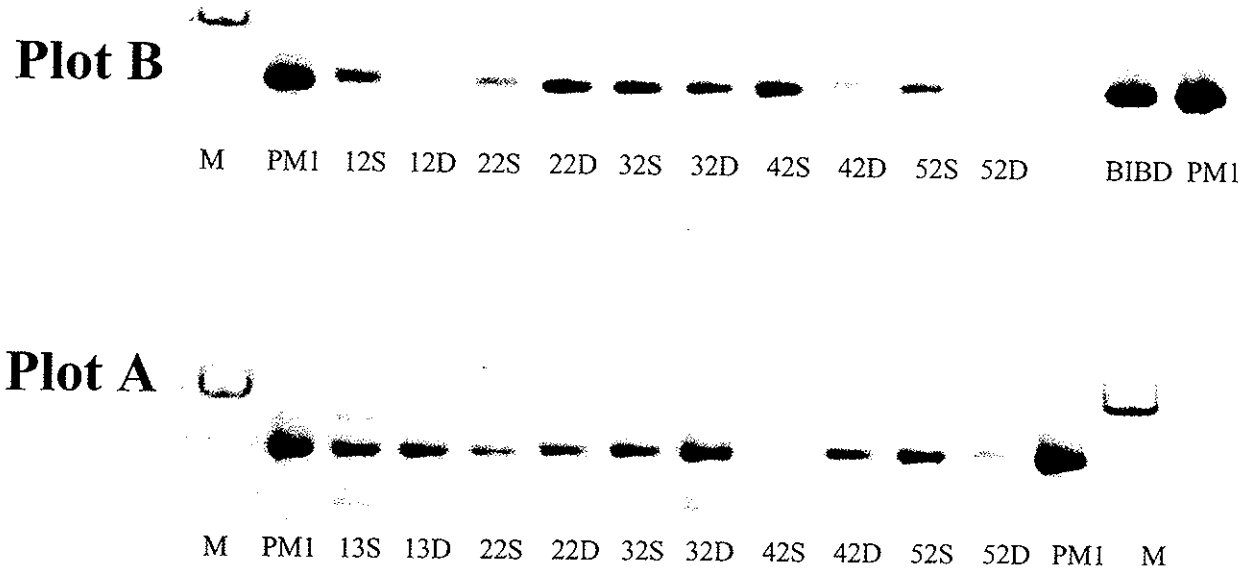

OXYGEN
Oxygen and PM1 (Plot B)
Deep Centerline
Oxygen Concentration (mg/L)
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
-9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5
Distance from Bottom of Oxygen Injection Bed (m)
0 days (Nov 9)
31 days (Dec 8)
66 days (Jan 11)
87 days (Feb 1)
120 days (Mar 6)
155 days (Apr 10)
190 days (May 15)

Figure 9. ITS analysis: PM1 presence in Plot B 24 days after injection of strain PM1 vs. Plot A (oxygen only)



Inverted images of SYBR Green-stained polyacryamide gels showing the PM1 ITS PCR amplicons. Lane1: PM1 ITS products (444bp) were obtained with PM1 specific primers in a final volume of 25 µl. PCR products were applied in 5-10 µl aliquots to wells in 5% polyacrylamide gels, and electrophoresed at 150V for 4 h in 1XTAE (Tris-acetate-EDTA, pH 8) running buffer.

Figure 10 a)

TaqMan Analysis: PM1 CFU per ml groundwater in Port Hueneme Plot B vs. Plot A as measured by Real-Time TaqMan PCR

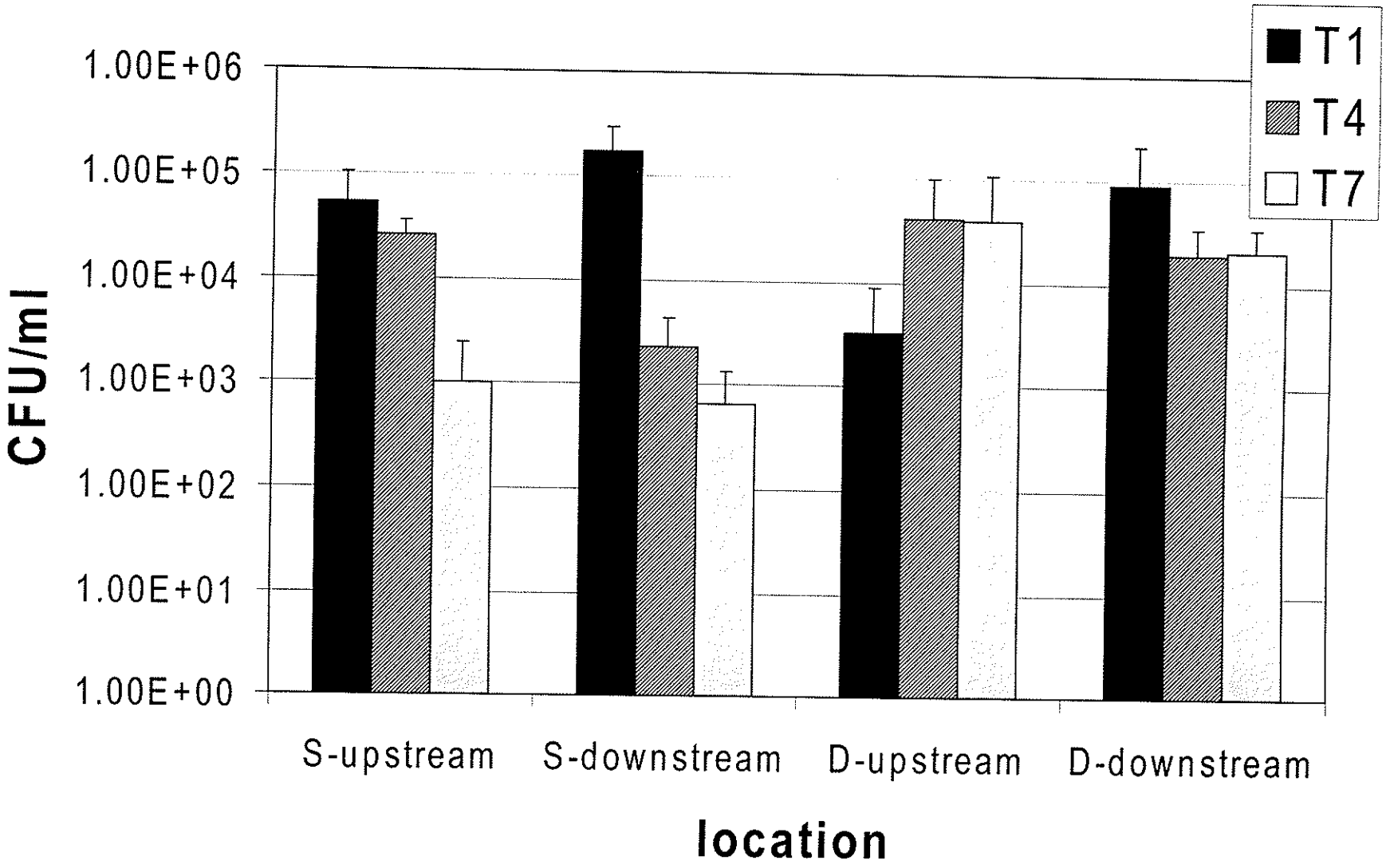


Figure 10 b)

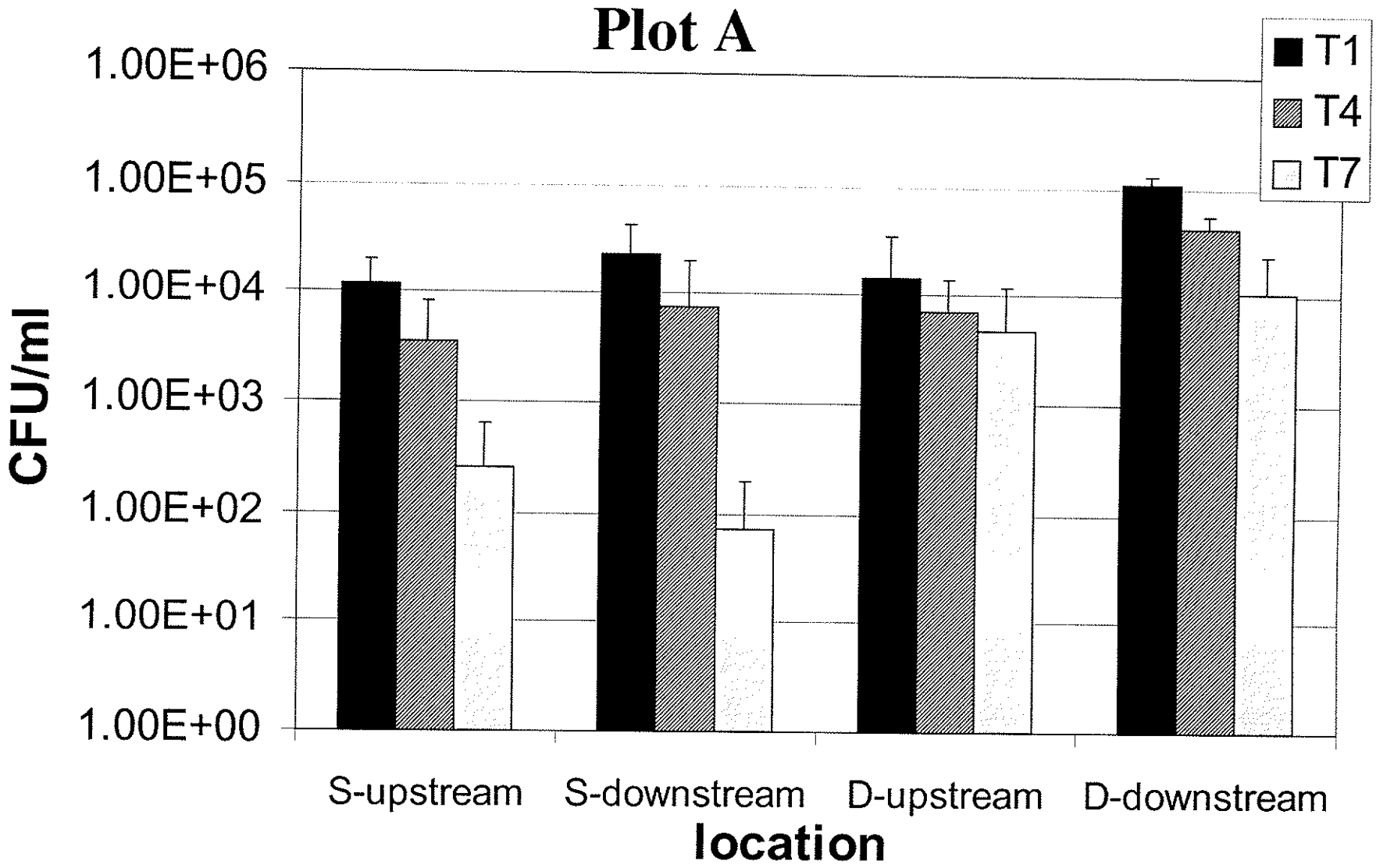
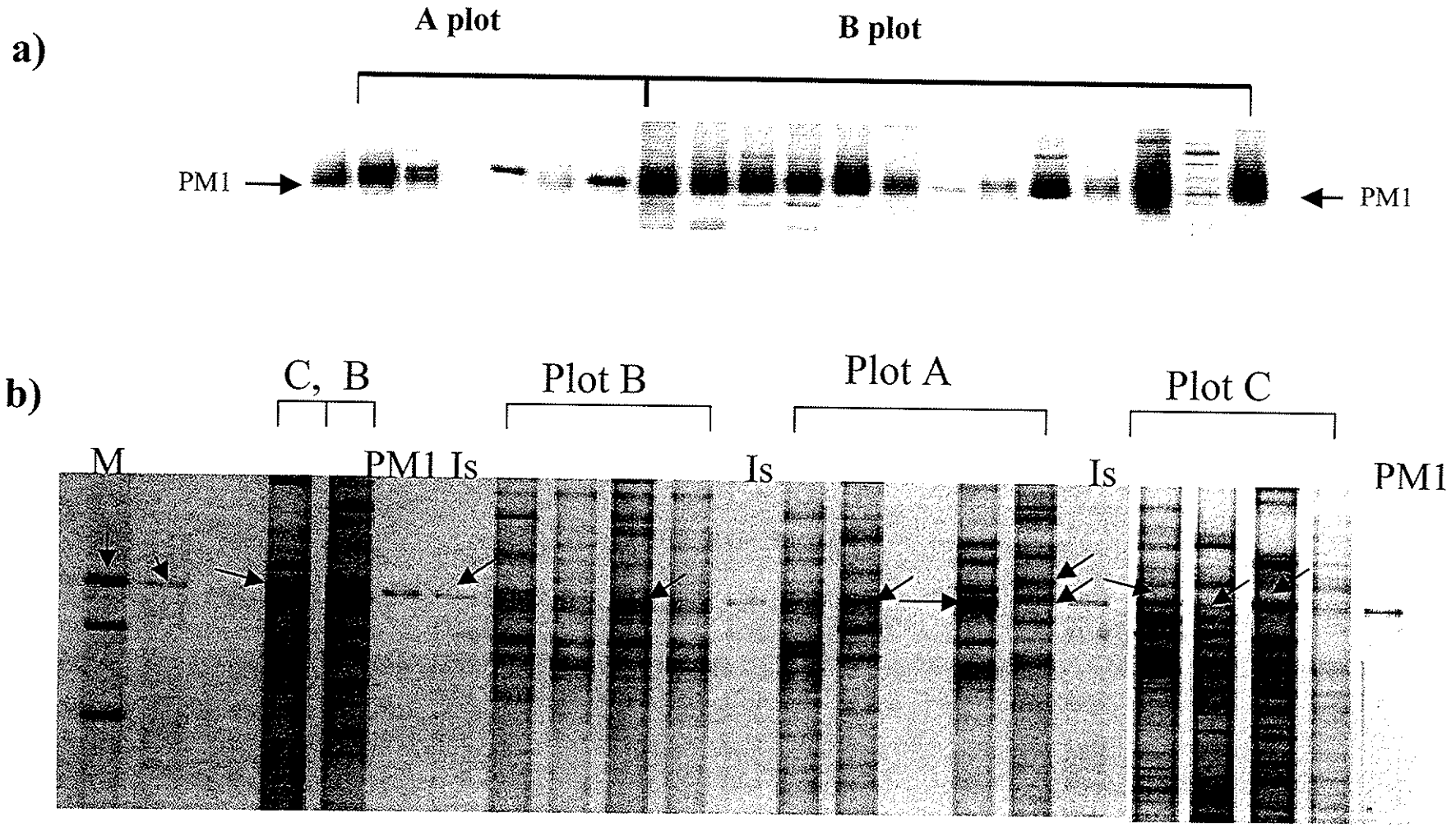
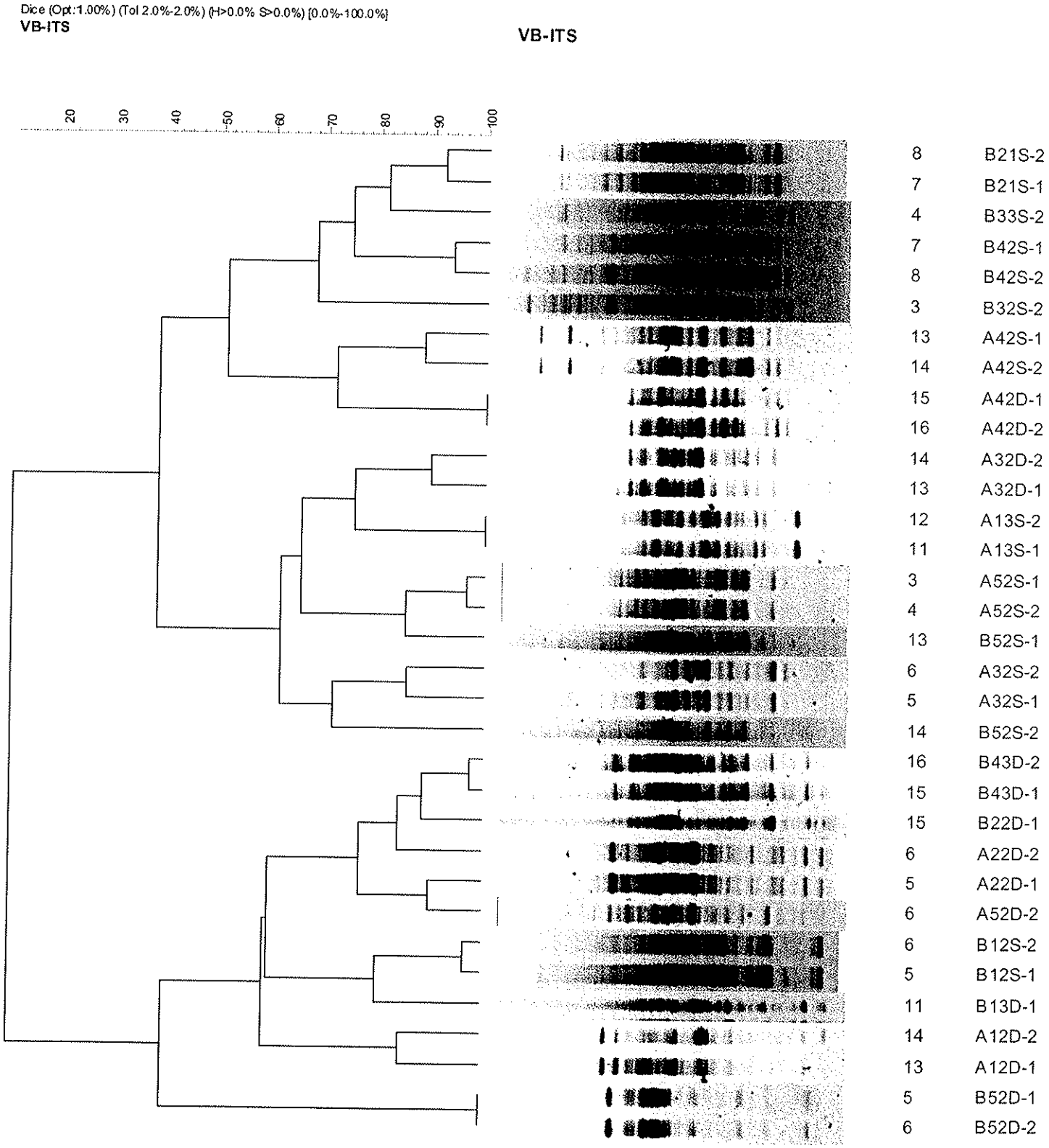

Plot A
T1
T4
T7
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
CFU/ml
S-upstream
S-downstream
D-upstream
D-downstream
location

Figure 11. DGGE with PM1 specific and universal bacterial primers reveal band similar in size to PM1 and to strain isolated from PH



Is = Port HuenemePM1-like isolate

Figure 12. Cluster diagram of ITS DNA fingerprints of groundwater microbial community.



Profiles were analyzed by the Dice coefficient and grouped with UPGMA. Barr indicates % ITS fingerprint similarities.

Table 1. PM1 presence in Port Hueneme as quantified by real-time TaqMan PCR

well	CBC-1	CBC-10	CBC-42	CBC-61-CS	CBC-60-CD	B-32-S	B-32-D	CBC-51	PHB4-1	PHA4-1
	← upstream							downstream →		
CFU/ml August 2000	<DL	<DL	<DL	<DL	$2.2x10^4$	$4.6x10^2$	$3 x10^4$	$1.9 x10^5$	$5.7 x10^3$	<DL
Distance from UCD plots (m)	800	649	303	40	40			474	623	737

DL = 180 CFU/ml groundwater